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PRINCIPAL INVESTIGATOR: Hasan N. Rajabi, Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

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**6. AUTHOR(S)**
Hasan N. Rajabi, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Dana-Farber Cancer Institute
Boston, MA 02115

E-Mail: hasan_rajabi@dfci.harvard.edu

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**13. ABSTRACT (Maximum 200 Words)**

Inactivation of retinoblastoma gene (*Rb*) is observed in several human cancers including those of the breast. A characteristic feature of many human cancers is the inability to maintain a terminal cell cycle arrest, whereas the *Rb* product (pRb) has been implicated in the maintenance of a terminal cell cycle arrest. However, in contrast to our knowledge of how pRb regulates proliferation in a cycling population, little is known how it maintains a permanent cell cycle arrest. The proposed studies are aimed at elucidating the molecular mechanism by which pRb accomplishes this task and plays the role of tumor suppressor of tumor formation. Our working hypothesis is that pRb, in cooperation with MyoD, participates in the transcriptional repression of one or more immediate early genes required for the induction of cyclin D1. And this event ultimately prevents the re-entry into the cell cycle, thus maintaining a terminal cell cycle arrest. To test this hypothesis myogenic differentiation has been used as model, because it represents a differentiation system in which pRb has been implicated in a terminal cell cycle arrest both in vitro and in vivo. In the past year I have discovered that: (1) The induction of Fra-1 and not any other immediate early genes is blocked following restimulation of differentiated myoblasts. (2) Ectopic expression of the cell cycle inhibitory protein p16, which bring about a cell cycle arrest distinct from a terminal cell cycle arrest, has no effect on expressions of both Fra-1 and cyclin D1. In an effort to further study the regulation of the Fra-1 gene I have created Fra-1 promoter reporter and its deletion mutants. Also constructed a retrovirus vector for ectopic expression of Fra-1 to establish a causal relationship between Fra-1 and cyclin D1. These results and reagents provide the basis upon which to discover the detailed mechanism by which pRb participates in a terminal cell cycle arrest.

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## Introduction:

A characteristic feature of most cancers is an increase in the percentage of proliferating cells, often referred to as mitotic index. Upon differentiation most cells in the body enter an irreversible terminal cell cycle arrest. Failure to maintain this growth-arrested state is thought to contribute significantly to the development of most forms of human cancer including those of the breast. The retinoblastoma protein (pRb) has been shown to participate in the maintenance of a terminal cell cycle arrest (12), however, the mechanisms by which it accomplishes this task are not understood. This is in contrast to the role of pRb in controlling proliferation in a cycling population, which is well characterized. The purpose of these studies described here are to elucidate the molecular mechanism by which pRb maintains a terminal cell cycle arrest. Initial focus is placed on the study on skeletal muscle differentiation since both *in vitro* and *in vivo* studies have clearly demonstrated a role for pRb in maintaining an arrested state following terminal differentiation of this tissue type (12, 17). Information gained from this analysis will then be applied to study of mammary gland differentiation. Inactivation of the retinoblastoma gene (*Rb*) is a common event in the development of several human cancers including those of breast. These studies thus likely have direct bearing of how loss of *Rb* contributes to the development of cancer.

## Body:

*Task 1: To establish various experimental systems to study terminal growth arrest*

Initially the genuine mouse myoblast line (C2C12) was utilized as an experimental tool, as these cells express both endogenous MyoD and pRb. Also, they respond differently to their cell culture conditions and can achieve either an irreversible growth arrest or a state of quiescence (reversible). To establish the condition for the irreversible growth arrest or quiescence, C2C12 cells were grown in 1% horse serum (for differentiation) or 0.5% FBS (for quiescence) containing media respectively. Cells were growth arrested for at least 72 hours in either of above media. Both differentiated or quiescence cells were serum stimulated with 20%FBS containing media to induce proliferation. The S-phase induction of re-stimulated cells was analyzed for by BrdU incorporation. Cells grown in condition known to cause differentiation (1%HS media) as described above did not show any S-phase induction whereas the cells grown to quiescence (0.5% FBS media) clearly incorporated BrdU upon serum re-stimulation indicating a S-phase induction (data not shown). These observations suggest that I have successfully established culture conditions to study an irreversible or reversible cell cycle arrest.

Next, I extended these observations to littermate matched $Rb^{+/+}$ and $Rb^{-/-}$ 3T3 mouse fibroblasts. These cells can be converted to myoblasts by infection with a MyoD encoding retrovirus. Use of this experimental system affords the ability to simultaneously assess the contribution of MyoD and pRb to a terminal cell cycle arrest. Initial efforts were placed on establishing the conditions for successful retroviral transduction to the 3T3 fibroblast cells, utilizing a virus encoding MyoD. The experimental conditions were established such that *Rb*-positive fibroblasts transduced with a MyoD-encoding retrovirus cultured under conditions known to induce myogenesis (1% horse serum containing medium for 72 hours),would not re-enter the cell cycle, but the same cells when infected with an 'empty' retrovirus do re-enter S phase upon restimulation. I found that indeed pRb and MyoD cooperate to prevent cell cycle re-entry of differentiated myoblasts following serum restimulation (data not shown). We feel that these observations may extend to other tissues, e.g., pRb may cooperate with a 'MyoD-like' factor to bring about a terminal cells cycle arrest in the breast.

*Task 2: To perform analysis of immediate early and delayed early gene expression*

Our working hypothesis is that the lack of cell cycle re-entry in pRb- and MyoD-positive myoblasts (following restimulation) is achieved by inhibiting the induction of one or more immediate early genes, which prevent the induction of cyclin D1, an event required for cell cycle re-entry. To test the validity of our hypothesis, we first used the genuine mouse myoblast C2C12 cells. Cells were grown under conditions known to cause irreversible arrest (differentiation) and then challenged with the addition of 20% FBS containing growth media. The expression of several immediate early genes was assessed by immunofuorescence. Among the immediate early genes analyzed only Fra-1 was not induced (see figure 1 in appendix). This lack of induction of Fra-1 suggests the possibility that Fra-1 may be the target of pRb and MyoD in their role of maintenance of a terminal cell cycle arrest.

Next, attempts were made to establish the cooperative role of pRb and MyoD in blocking the induction of Fra-1 following restimulation of differentiated myoblasts. *Rb* positive and negative fibroblasts were transduced with MyoD expressing or empty retrovirus. Cells were then restimulated and harvested at various time points and the expression of several immediate early genes analyzed by Western blot analysis. The *Rb* positive fibroblasts transduced with MyoD did not show the re-induction of cyclin D1 or Fra-1 upon serum stimulation. Other immediate early genes, e.g., Fra-2 or c-Jun, showed a clear and significant induction. By contrast, in *Rb*-deficient myoblasts cyclin D1, Fra-1 and other immediate early genes were induced (See Figure. 2 in appendix). To analyze the expression of c-Fos protein in *Rb*—MyoD dependent manner the cell lysates from early time points were used in Western blot. Here too c-Fos protein did not show any inhibition of induction upon serum stimulation. Also the expression of MyoD in both *Rb* +/+ and *Rb* -/- cell lysate is found to be at comparable level as seen in the other Western blot. These observations suggest that Fra-1 among other immediate early or delayed early genes, shows a lack of induction upon serum stimulation in an pRb- and MyoD- dependent manner. These results, together with our analyses of C2C12 myoblasts (see above), further support the hypothesis that cooperative function of MyoD and pRb specifically inhibit the induction of immediate early gene Fra-1, which in turn prevents the induction of the essential cell cycle protein cyclin D1, thus allowing the maintenance of a terminal cell cycle arrest.

One of my working hypotheses is that the role of pRb in maintaining a terminal cell cycle arrest is distinct from its participation in a mid G1 cell cycle arrest. Consistent with this notion others have recently separated these two functions of pRb genetically (11). Further, we hypothesize that an ultimate target of pRb during a terminal cell cycle arrest is cyclin D1. This is in contrast to the mid G1 arrest brought about by pRb where cyclin D1 expression is not affected. We have confirmed the above hypothesis using *Rb*+/+ fibroblasts transduced with retrovirus encoding MyoD, the cdk inhibitor p16 or vector. The transduced fibroblasts were treated with differentiation conditions for 72 hours and followed by re-stimulation with 20% fetal bovine serum containing media. Cells expressing either MyoD or p16 failed to re-enter the cell cycle as determined by FACS analysis (data not shown), whereas vector infected cells, as expected, progressed into S phase. By contrast, Fra-1 and cyclin D1 was not induced only in MyoD expressing cells as observed in Western blots (see Figure 3 in appendix). Importantly, in the cells infected with the p16-encoding virus or vector control, both Fra-1 and cyclin D1 proteins were induced following re-stimulation. These observations suggest that the pRb target cyclin D1 during a terminal cell cycle arrest and its function in terminal cell cycle arrest and a mid G1 arrest are distinct.

In contrast, these genetically defined cells (*Rb*+/+ or *Rb*-/- 3T3) when treated only with the conditions known to cause differentiation (without being transduced with either MyoD or retrovirus vector) followed by

restimulation, shows a clear induction of Fra-1 and cyclin D1. Importantly the temporal expression of Fra-1 is prior to cyclin D1 expression (see figure 4 in appendix), which is consistent with our hypothesis that Fra-1 can be a participant in the induction of cyclin D1. Also the earlier induction of Fra-1 expression indicates that Fra-1 is more proximal to the affect of MyoD and pRb during a terminal cell cycle arrest.

With regard to the experiments outlined above it is noteworthy that while several immediate early genes have been implicated in the induction of cyclin D1 (3,5,9,15) only one implicates Fra-1 (1). Thus, I sought to causally connect Fra-1 expression with cyclin D1 induction using the myoblasts system. As we know from reports of previous other investigators that the Fra-1 protein does not have a Transactivation Domain (TAD) to be able to function alone (4,14,16). And it is been shown that Fra-1 protein to be functionally active needs to heterodimerize with c-Jun family proteins and cannot homodimerize with any other c-Fos family protein (6,7,10,14). We also know that c-Fos and c-Jun family proteins are partners in AP-1 complex formation for to participate in various cell cycle regulatory activities (2,6,8). Since Fra-1 but no other immediate early genes show any inhibition upon serum stimulation in *Rb*-MyoD dependent manner when grown in conditions known to cause differentiation. Therefore, an ectopic expression of Fra-1 may be able to prevent the effect of *Rb* positive cells to undergo terminal cell cycle arrest in the presence of MyoD and will cause the cell cycle re-entery upon serum restimulation.

In order to achieve a more solid link between Fra-1 expression and cyclin D1 induction, I have constructed an adenovirus capable of directing the expression of HA-tagged mouse Fra-1. This adenovirus will be used to ectopically express the Fra-1 in the *Rb*+/+ and *Rb*-/- cells along with the MyoD expression. Here ectopic Fra-1, but not vector control, in *Rb*+/+ fibroblasts undergoing differentiation might allow the induction of cyclin D1 upon serum restimulation. Such a result will provide evidence that Fra-1 participates in the induction of cyclin D1 expression (under circumstances where a terminal cell cycle arrest is not achieved). As the adenovirus vectors can ectopically express the protein of interest in differentiated myoblasts, it easily overcomes the limitations of the retrovirus mediated expression of Fra-1, which needs a dividing cells for expression of protein as well as the continued expression of Fra-1 which may even prevent the cells to enter the terminal cell cycle arrest. This approach will allow linking Fra-1 to the expression of cyclin D1, thereby supporting the hypothesis that it is the block to Fra-1 induction leads to inhibition of cyclin D1 expression following re-stimulation of arrested myoblasts.

*Task 3: To characterize and elucidate the molecular involvement of retinoblastoma protein in terminal growth arrest*

Our preliminary data with defined genetic background myoblast suggests that the Fra-1 gene is a primary target for pRb and MyoD during a terminal cell cycle arrest. A major goal in this line of research is to determine how, mechanistically, pRb and MyoD cooperate to prevent the induction of Fra-1 following restimulation of myoblasts cultured under differentiation conditions. We suspect that somehow pRb and MyoD actions converge upon the Fra-1 promoter to achieve such sustained silenced mode.

As reported last year, I have generated a mouse Fra-1 promoter reporter construct, identical to the physiological promoter (wild type) except being fused upstream to luciferase reporter in order to understand the possible role of pRb and MyoD on Fra-1 promoter in preventing the activation of Fra-1. Numerous investigators have studied the regulation of the Fra-1 gene. Regulation of this gene is complex, as both the 5' flanking sequence and intron-1 have been implicated in its regulation (13). Thus, I have constructed a Fra-1 promoter luciferase reporter construct where both 5'UTR and intron-1 were cloned upstream to the reporter gene as closely identical

to the physiological promoter and designated it as wild type promoter. I have established that the wild type Fra-1 promoter reporter responds faithfully to activation in transient transfection of asynchronous dividing C2C12 myoblast cells. Considering the functional integrity of wild type promoter we have further generated various deletion mutants from this full-length wild type promoter of Fra-1 (See Figure 5 in appendix) and found that these deletion mutants though retain the functional integrity, but to a very low level compared to wild type promoter construct. Further, these Fra-1 promoter reporter constructs will assist to locating the conserved responsive elements, which may be regulated by the presence of *Rb*-MyoD in maintaining terminal cell cycle arrest.

The integrity and functional viability of Fra-1 promoter reporter constructs were verified by transiently transfecting them in C2C12 cells. The goal was to find out whether this extra chromosomal promoter reporter construct responds in similar manner to the one seen from the endogenous promoter activation leading to induction of Fra-1 protein expression. To accomplish this task, C2C12 myoblasts were transfected either with full-length or various deletion mutants of promoter reporter construct. The transfected C2C12 cells grown to differentiation conditions (DMEM plus 1% horse serum) and re-stimulated with 20% fetal bovine serum containing media did not show any significant induction of luciferase activity. In contrast the transfected cells grown to quiescence (DMEM plus 0.5%FBS) for the same duration showed a clear and significant induction upon serum stimulation. (See figure 6 in appendix). Interestingly, this positive induction in quiescence treated cells and lack of induction in differentiation treated cells was similar for various deletion mutants as well (See figure 7 in appendix and data not shown), but all the deletion mutants had nearly 10 fold lower activation than that of the full-length promoter (See figure 7 in appendix and data not shown). This difference in promoter activity indicates that maybe the activation of Fra-1 promoter involves multiple regions of the promoter. Also these results indicate that transiently transfected extra chromosomal Fra-1 promoter reporter constructs faithfully reflect the functional outcomes to growth conditions of host cells.

As we reported last year the possible technical constrains related to the transient transfection approach for Fra-1 promoter reporter constructs analysis in our study. Therefore, these various promoter reporter constructs have been stably integrated into litter matched, defined genetic background of *Rb* +/+ and *Rb* -/- cells derived from wild type and *Rb*-deficient mouse embryo fibroblasts. Both clonal lines or pooled population for full-length promoter and all the deletion mutants have been selected. The functional integrity of these promoter reporter lines and the pooled population has been verified by their luciferase activity. Thus circumventing the technical constrains of transient transfection, these clonal lines of Fra-1 promoter reporter were used for subsequent studies to assist in understanding the mechanism of Fra-1 promoter regulation.

Continuing our effort to establish the cooperative function of pRb and MyoD on Fra-1 promoter, a pooled population of Rb+/+ or Rb-/- cells containing stably integrated Fra-1 full length promoter reporter was transduced with MyoD or empty retrovirus vector. These transduced cells were subjected to differentiation conditions and challenged with serum stimulation for a growth induction as described earlier. The cells were harvested at various time points after serum stimulation and promoter activity was analysed by Luciferase activation. The MyoD expressing myoblasts did not show any induction of Fra-1 promoter (see figure 8 in appendix), whereas the empty vector infected cells showed a clear up-regulation in Luciferase activity. In contrast the Rb-/- cells transduced either with MyoD or vector retrovirus showed a similar level of promoter activation at various time points. Indeed the promoter reporter activity is in full agreement with the Fra-1 and Cyclin D1 protein expression, thus further confirming a clear cooperative function between pRb and MyoD in Fra-1 promoter regulation.

The above observations of Fra-1 promoter reporter activation and inhibition of induction further support our hypothesis that the MyoD may be directly binding to the Fra-1 promoter. In order to validate our hypothesis that direct binding of MyoD is crucial event in terminal cell cycle arrest a sequence analysis of the Fra-1 promoter was carried out. Using Bayesian block alignment phylogenetic analysis (see figure 9 in appendix) both for 5'upstream and intron-1 sequences from mouse, human and rat revealed a conserved E-box elements in these regions. Such high sequence conservation encouraged us to investigate the possibility of direct binding of MyoD to Fra-1 promoter in pRb and MyoD dependent manner. To confirm the likely binding of MyoD to Fra-1 promoter, a Chromatin immunoprecipitation (ChIP) assay was carried out. First, the genuine mouse myoblast C2C12 cells were used to determine if MyoD is actually binding to the Fra-1 promoter. Here these cells were treated with conditions known to cause differentiation as described earlier and serum stimulated. Cells were cross-linked with 1% Formaldehyde, collected and washed and then subjected to sonication Aim was to generate chromatin fragments in range of 500 to 1000 base pairs in length. Cleared lysate was subjected to immuno-precipitation using the antibody against MyoD (C20, SanatCruz Biotech) and co-precipitated chromatin released by reversing the cross linking at $65^0$C over night and DNA was purified by Qiagen columns for PCR amplification. One microliter of each sample was used as template for PCR amplification of Fra-1 promoter fragment from the intron-1 region with conserved MyoD binding sites. In addition, primer pairs predicated to amplify DNA fragment 3000 base pair downstream to first exon used as negative control. Also a primer pair for the known MyoD responsive promoter of late differentiation marker gene the muscle creatine kinase (MCK) was used as positive control. As predicted DNA template generated through ChIP from both the differentiated and stimulated C2C12 cells clearly showed a PCR amplification of Fra-1 promoter fragment (see figure 10 in appendix), thus supporting our hypothesis that MyoD is directly binding to Fra-1 promoter both during differentiation and continued to be present even after stimulation.

Next, to further demonstrate that the pRb and MyoD play a cooperative function in inhibition of induction of Fra-1 promoter in maintaining terminal cell cycle arrest, the defined genetic background (Rb+/+ and/or Rb-/- 3T3 cells) myoblasts with ectopic MyoD were used for ChIP analysis. As described earlier the Rb+/+ 3T3 cells were transduced with MyoD or empty vector and grown under conditions known to cause their differentiation to myoblast. Cells were harvested before and after serum stimulation for ChIP assay identically as described above for genuine myoblast (C2C12 cells). The DNA templates generated from ChIP assay with anti-MyoD antibody were used for PCR amplification of Fra-1 promoter region. The ethidium bromide staining of 1% agrose gel resolved PCR products clearly shows the presence of Fra-1 promoter specific fragment in MyoD transduced *Rb*+/+ cells whereas no bands were visible in vector infected cells. The PCR amplification for a fragment of MCK promoter region was used as positive control, to which MyoD is known to directly bind during differentiation. A non-specific DNA fragment for HSC70 gene and sequences 3000 base pairs downstream to MyoD binding site in Fra-1 promoter were used as negative control for anti-MyoD antibody, which clearly scored negative for PCR amplification (see figure 11 in appendix). These observations with defined genetic background myoblasts strongly support our hypothesis that pRb somehow regulate the MyoD to converge upon Fra-1 promoter to maintain a terminal cell cycle arrest.

In order to further support our hypothesis that pRb and MyoD actions somehow converge upon Fra-1 promoter and direct binding of MyoD is crucial event in terminal cell cycle arrest, a mutational analysis of the MyoD binding site (E-box elements) in Fra-1 promoter was carried out. As evident from the Bayesian block alignment analysis of the Fra-1 promoter the E-box elements (Myo D responsive elements) as well as AP-1 binding sites (growth stimulus responsive elements) were localized in conserved sequence stretch. To confirm our hypothesis, we generated three different point mutations of putative E-box elements present in intron-1 region of Fra-1 promoter reporter. The mutations were made by site directed mutagenesis, out of which two are single

site mutation and one mutant has both the E-box elements changed to a non functional E-box element. The idea behind generating such point mutation was to see if the loss of E-box element in Fra-1 promoter directly reflects to loss of inhibition of induction in presence of both pRb and MyoD, which is otherwise seen in direct correlation. To establish the above hypothesis, a pooled population of wild type promoter and two of the E-box mutant (E-box-1 and E-box-2 single mutants) clonal lines in pRb+/+ cells were transduced with MyoD expressing or vector retrovirus. As described earlier, cells were treated with the conditions known to cause differentiation and serum stimulated to check the Fra-1 promoter activation at various time points by luciferase reporter assay. The wild type promoter and E-box-1 (distal from conserved AP-1 sites) showed a significant inhibition of induction in presence of MyoD and activation in vector transduced cells (see figure 12 LUC assay for E-box mutant). However, the E-box-2 mutant (proximal to conserved AP-1 sites) showed similar level of activation at various time points after serum stimulation, in both MyoD or vector infected cells. This result further supports that MyoD may be directly binding to the E-box element in Fra-1 promoter in order to inhibit the induction. Moreover the loss of inhibition in E-box mutation (proximal to AP-1 site) also indicates that either MyoD is somehow involved in chromatin remodeling of Fra-1 promoter or helping some other transcriptional regulators to converge at promoter.

Together, these observations of cooperative role played by pRb and MyoD in maintaining a terminal cell cycle arrest, it becomes utmost important to access the physiological relevance of pRb and MyoD function. In order to analyze this, the ongoing experiments are to carry out an *in-situ* hybridization on skeletal muscles sections generated from defined genetic background (Rb+/+ or -/-) E14.5 day mouse embryo. As we know from our earlier observations in the lab that the *Rb*-/- mouse embryos die at E13.5-E14.5 days and show defects in skeletal muscles differentiation and aberrant cell proliferation, which is not rescued by the loss of N-ras. Thus keeping this separable function of *Rb* in differentiation and terminal cell cycle arrest we will check the levels of cyclin D1 and Fra-1 messages in those proliferating skeletal muscle cells with large endoreduplicating nuclei using riboprobe against either of them. We have already generated E14.5 embryos and longitudinal sections of skeletal muscles are prepared for this analysis. We hope this study will further strengthen our hypothesis and support the cooperative role played by pRb and MyoD in maintaining the terminal cell cycle arrest in a physiological relevant manner.

These research accomplishments so far support the hypothesis being tested in this proposal. Further, the established cell culture conditions along with inclusion of various other analytical tools assisted us to propose how mechanistically pRb and MyoD may be cooperating to maintain a terminal cell cycle arrest. Further, they form a working foundation on which to test the possibility that a similar mechanism is employed by other cell types, e.g. mammary epithelial cells, to maintain an arrested state— also a goal of future research.

This line of investigation provides an excellent training in basic molecular biological techniques pertaining to cell cycle and differentiation— two key aspects to the study of breast cancer. It also taught me how to carefully design an experiment to test a hypothesis.

## Key research accomplishments:

- Cell culture conditions established for the defined genetic system generated from *Rb*+/+ and *Rb*-/- mouse fibroblast allowing the study of a terminal cell cycle arrest
- Condition for efficient retroviral infection for MyoD and p16 to the defined genetic system of mouse fibroblasts has been established

- Established that terminal cell cycle arrest is distinct from the well characterized mid G1 arrest brought about by the cdk inhibitor p16
- Determined that the Fra-1 induction precedes the cyclin D1 induction in my defined genetic system of *Rb*+/+ and *Rb*-/- mouse fibroblasts, suggesting that Fra-1 participates in the induction of cyclin D1 and that Fra-1 gene regulation is likely the target of pRb and MyoD action in maintenance of a terminal cell cycle arrest.
- Determined Fra-1 and not any other immediate early genes is the target of pRb and MyoD action during a terminal cell cycle arrest, suggesting some degree of specificity in the mode of action of pRb and MyoD in maintaining a terminal cell cycle arrest.
- Constructed an Adenovirus and Retrovirus vector for ectopic expression of Fra-1 to establish a causal relationship between Fra-1 and cyclin D1 expression.
- Determined that the activity of a Fra-1 promoter reporter construct faithfully recapitulates the expression of the endogenous gene during restimulation of quiescent and differentiated myoblasts.
- Established the Fra-1 promoter reporter stable clonal lines in pRb defined genetic background 3T3 fibroblast.
- Determined that the Fra-1 promoter reporter line of *Rb*+/+ cells show a clear inhibition of induction in presence of MyoD.
- Determined the presence of MyoD on Fra-1 promoter by ChIP analysis of genuine myoblast defined genetic background Myoblast both after differentiation and stimulation.
- Generated E-box mutation carrying clonal line in defined genetic background and determined the loss of one of the MyoD binding loses the property on inhibition of induction.
- Generated E14.5 day old mouse embryo for in situ hybridization analysis for Fra-1 and cyclin D1 in Rb-/- and Rb+/+ state.

## Reportable outcomes:

- Fra-1 promoter reporter constructs: full length and various deletion mutants of Fra-1 promoter reporter cloned upstream to Luciferase gene
- Rat Fra-1 coding gene is cloned in to pBabe-puro retrovirus expression vector
- Cell lines: Fra-1 promoter reporter constructs stably transfected to cell lines of Rb+/+ and Rb-/- mouse fibroblast and clonal lines are created
- Murine Fra-1 coding gene with HA-tag is cloned into adenovirus vector and expressed in 293T cells.
- Chromatin immunoprecipitation (ChIP) analysis: localize MyoD on the E-box elements in Fra-1 promoter both in genuine myoblast (C2C12) and MyoD transduced pRb+/+ 3T3 fibroblast under differentiation and stimulation condition.
- Site directed mutagenesis of conserved E-box element in Fra-1 promoter: established a clonal line for the mutants.
- Lack of inhibition of induction in one of the E-box mutant indicating a direct binding of MyoD on Fra-1 promoter.

## Conclusions:

One of the characteristic features of cancer is the inability to maintain a terminal cell cycle arrest. *Rb* has been reported to be inactivated in various human cancers including those of the breast. Also the pRb has been implicated in maintaining the terminal cell cycle arrest, though the mechanism by which it accomplishes this is not known in contrast to a well-understood role in regulation of proliferation in cycling cells. Our studies

supported by this fellowship to date have been directed towards developing a system to study mechanistically how pRb participates in maintaining a terminal cell cycle arrest. My data suggest that pRb maintains a terminal cell cycle arrest by specifically blocking the expression of immediate early gene Fra-1, which in turn is responsible for the lack of induction of cyclin D1—otherwise an essential event required for re-entry of cells into the cell cycle. I have provided evidence that this mode of action of pRb is distinct from its well-characterized ability to mediate a mid G1 arrest. Importantly, I have developed a Fra-1 promoter reporter that behaves in a manner similar to the endogenous gene and successfully recapitulate the expression patterns of endogenous gene. Further using the ChIP assay for defined genetic background fibroblast with ectopic MyoD and genuine mouse myoblast C2C12 cells gave me a very convincing data of MyoD directly binding to Fra-1 promoter. It also indicated the direct binding of MyoD may be a critical factor in likely mechanism of terminal cell cycle arrest. The results from stably integrated wild type Fra-1 promoter reporter and the E-box mutant cell lines further confirmed the direct binding of MyoD on Fra-1 promoter for its regulation where the mutant dissimilar to wild type promoter reporter loses the inhibition of induction. Specifically, it suggests the means by which pRb and MyoD cooperate to maintain the Fra-1 in a silenced state following restimulation of differentiated myoblasts. The important observation is likely to be generated from *in-situ* hybridization of mouse embryo, which is ongoing and will support our hypothesis with the physiological significance of pRb and MyoD roles in terminal cell cycle arrest. Significantly the results obtained so far, provide a framework on which the study of a terminal cell cycle arrest can be extended to other cell types. Also it will help to find out the likely mechanism of pRb action in the suppression of tumor formation.

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## 18. Appendix:

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**Figure 12**

## Figure Legends:

**Figure 1.** Fra-1 is the only immediate early or delayed early gene getting specifically inhibited from induction in genuine mouse myoblast line C2C12 after serum stimulation of these differentiated myobalsts. (A-H) C2C12 cells incubated under conditions that induce myogenesis (1%HS) (A-D) or cause quiescence (E-H). Differentiated and quiescence cells were stimulated with 20%FBS containing media for 4 hours (B, D, F and H). Differentiated myoblast that had not (A) and had (B) been restimulated were then fixed, permiablized and stained with antibody to Fra-1 (rhodamine, Red) or myosin heavey chain (MHC; marker of muscle differentiation; FITC, green) and counterstained with DAPI to visualize nuclei (C and D). C2C12 rendered quiescent without (E) or with (F) restimulation were stained with same antibody to Fra-1 (rhodamine) or counterstained with DAPI (G and H).
Similarly (I-P) was analyzed as in A-H, except antibody to c-Fos was used and C2C12 cells were stimulated for 2 hours.

**Figure 2.** Fra-1 and cyclin D1 proteins are not induced following restimulation of *Rb*+/+ 3T3 mouse fibroblasts with MyoD after getting differentiated (1% Horse serum containing media) and Fra-1 is the only immediate early or delayed early gene getting specifically inhibited from induction as shown in western blot, but shows a clear induction in cells infected with vector or in the *Rb* -/- fibroblasts with MyoD. These differentiated myoblasts were stimulated for 8 hours in presence of 20% fetal bovine serum containing media. Cells were harvested in lysis buffer with protease inhibitors (50 mM Tris-Cl pH 8.0, 200 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 25 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 250 μM phenylmethylsulfonyl fluoride, 10 μg of Leupeptin per ml and 10 μg of aprotenin per ml). Equal amount of whole cell lysates were loaded in 10% SDS-polyacrylamide gel and transferred to Polyvinylidine difluoride membrane, probed with Fra-1 (N-17, SantaCruz Biotech) or cyclin D1 (AB-3, Neomarker) antibody, c-Fos, c-Jun, Fra-2 and MyoD antibody (SantaCruz Biotech Inc., USA).

**Figure 3.** Ectopic expression of p16 has no effect on the induction of cyclin D1 or Fra-1 proteins in differentiated (1% Horse serum containing media) *Rb*+/+ 3T3 mouse fibroblasts upon serum stimulation. These differentiated myoblasts were stimulated for 4 hours with 20% fetal bovine serum containing media. Cell lysates were prepared as described and equal amount of whole cell lysates were loaded in 12% SDS-polyacrylamide gel and transferred to Polyvinylidine difluoride membrane, western blot for p16 was carried out using ZJ-11 mouse monoclonal antibody.

**Figure 4.** Schematic diagram of Fra-1 promoter reporter construct and its deletion mutants generated from the full length Fra-1 promoter region.

**Figure 5.** Fra-1 promoter reporter construct (designated as close to wild type promoter) is transfected into C2C12 myoblast cells followed by treatment to conditions causing differentiation (1% Horse serum containing media) or irreversible quiescence (0.5% Fetal bovine serum containing media) before stimulation with 20% fetal bovine serum containing media at various time points. Luciferase reporter activation was analysed for Fra-1 promoter activity.

**Figure 6.** Deletion mutants of Fra-1 promoter reporter construct containing various regions of Fra-1 promoter was transfected to C2C12 myoblast cells followed by treatment to conditions causing differentiation (1% Horse serum containing media) or quiescence (0.5% Fetal bovine serum containing media) before stimulation with

20% fetal bovine serum containing media at various time points. Luciferase reporter activation was analysed for Fra-1 promoter activity.

**Figure 7.** Comparison of Fra-1 promoter reporter (WT) with its deletion mutants shows a high activation of full-length promoter compare to deletion mutants upon serum stimulation in both differentiation condition or quiescence condition treated C2C12 myoblast cells.

**Figure 8.** Fra-1 promoter reporter construct (designated as close to wild type promoter) stably integrated into Rb+/+ or Rb-/- fibroblasts. A pooled population of integrated stable line transduced with MyoD expressing retrovirus followed by treatment to conditions causing differentiation (1% Horse serum containing media). These myoblasts were stimulated with 20% fetal bovine serum containing media. Luciferase reporter activation was analysed at various time points after re-stimulation for Fra-1 promoter activity.

**Figure 9.** Bayesian block phylogenetic alignment of Fra-1 promoter sequences from human and mouse to check the conserved regions between two. This lead us to design the E-box mutant of the Fra-1 promoter to assist in understanding the possible mechanism of pRb and MyoD mediated regulation of Fra-1 promoter.

**Figure 10.** Chormatin Immunoprecipitation (ChIP) assay for differentiated genuine mouse myoblast cells using MyoD antibody (C-20, SantaCruz Biotech) to localize MyoD on Fra-1 promoter. The primer pair for MCK promoter was used as positive control and primers designed for 3000 base pair down stream of exon-1 was used as negative control for MyoD binding. Also a non-specific gene HSC-70 was used as another negative control.

**Figure 11.** Chormatin Immunoprecipitation (ChIP) assay for differentiated Rb+/+ mouse fibroblasts turned myobalsts using MyoD antibody (C-20, SanatCruz Biotech) to localize MyoD on Fra-1 promoter. The primer pair for MCK promoter was used as positive control and primers designed for 3000 base pair down stream of exon-1 was used as negative control for MyoD binding. Also a non-specific gene HSC-70 was used as another negative control.

**Figure 12.** Fra-1 promoter reporter constructs (designated as wild type, E-box-1 and E-box-2 mutants) stably integrated into Rb+/+ or Rb-/- fibroblasts. A pooled population of integrated stable line transduced with MyoD expressing retrovirus followed by treatment to conditions causing differentiation (1% Horse serum containing media). These myoblasts were stimulated with 20% fetal bovine serum containing media. Luciferase reporter activation was analysed at various time points after re-stimulation for Fra-1 promoter activity.

# Immediate early gene Fra-1 is inhibited from induction in differentiated genuine mouse myoblast upon serum stimulation

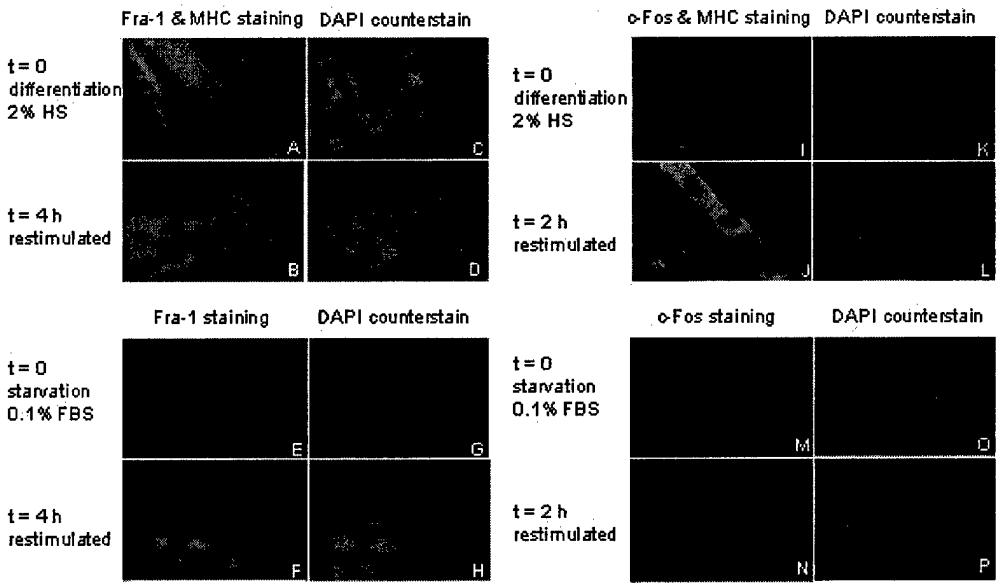



Figure 1

**Fra-1 is the only immediate early gene being inhibited from the induction upon serum stimulation in Rb+/+ fibroblasts**

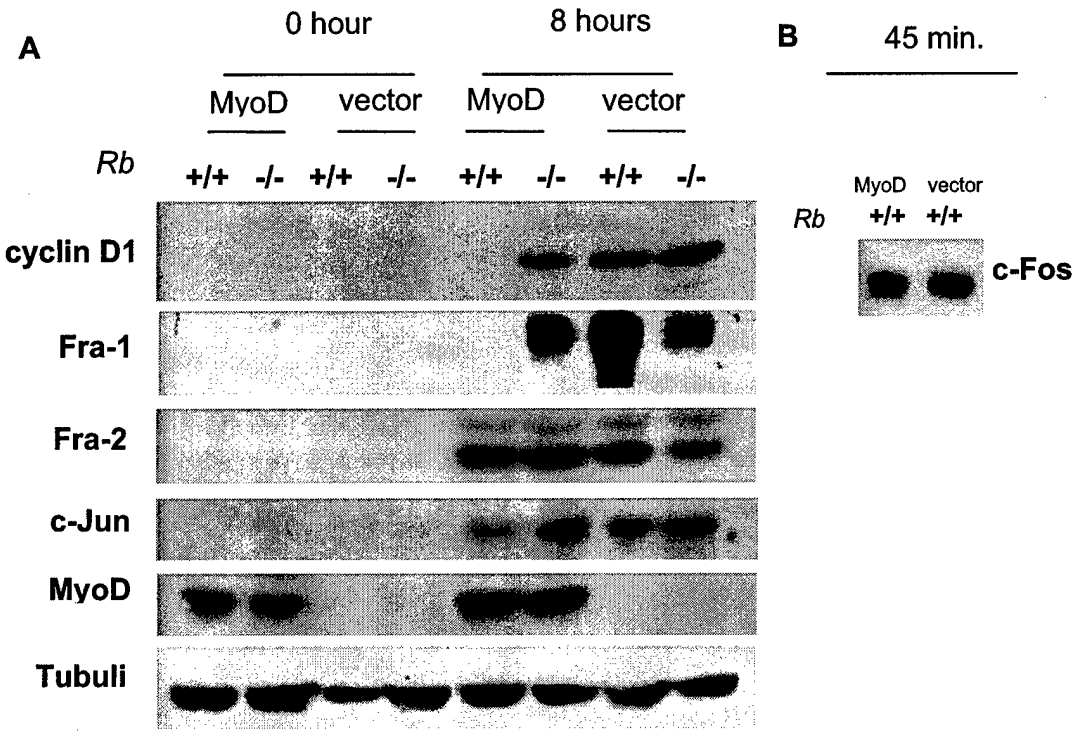



Figure 2

**Terminal cell cycle arrest mediated by the cooperation of pRb and MyoD is distinct from mid G1 arrest that mediated by p16**

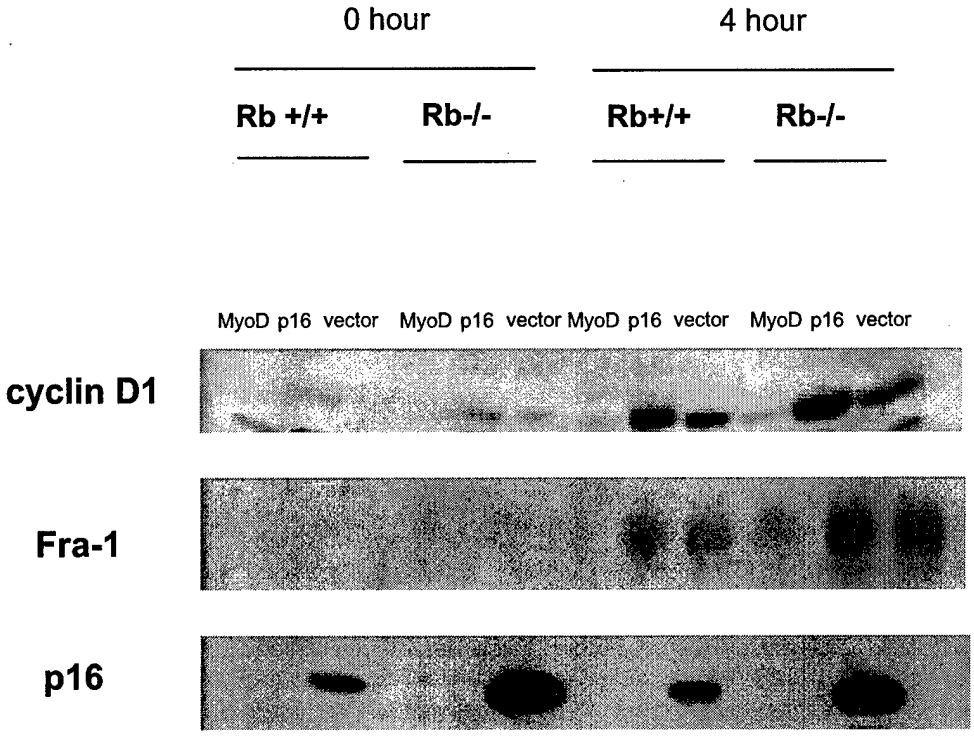



Figure 3

**Temporal expression pattern of Fra-1 and cyclin D1 is consistent with the notion that Fra-1 participates in the induction of cyclin D1**

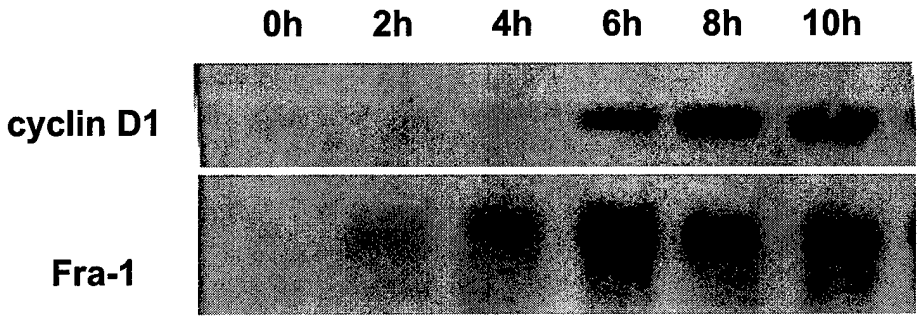



Figure 4

## Schematic diagram of Fra-1 promoter reporter constructs

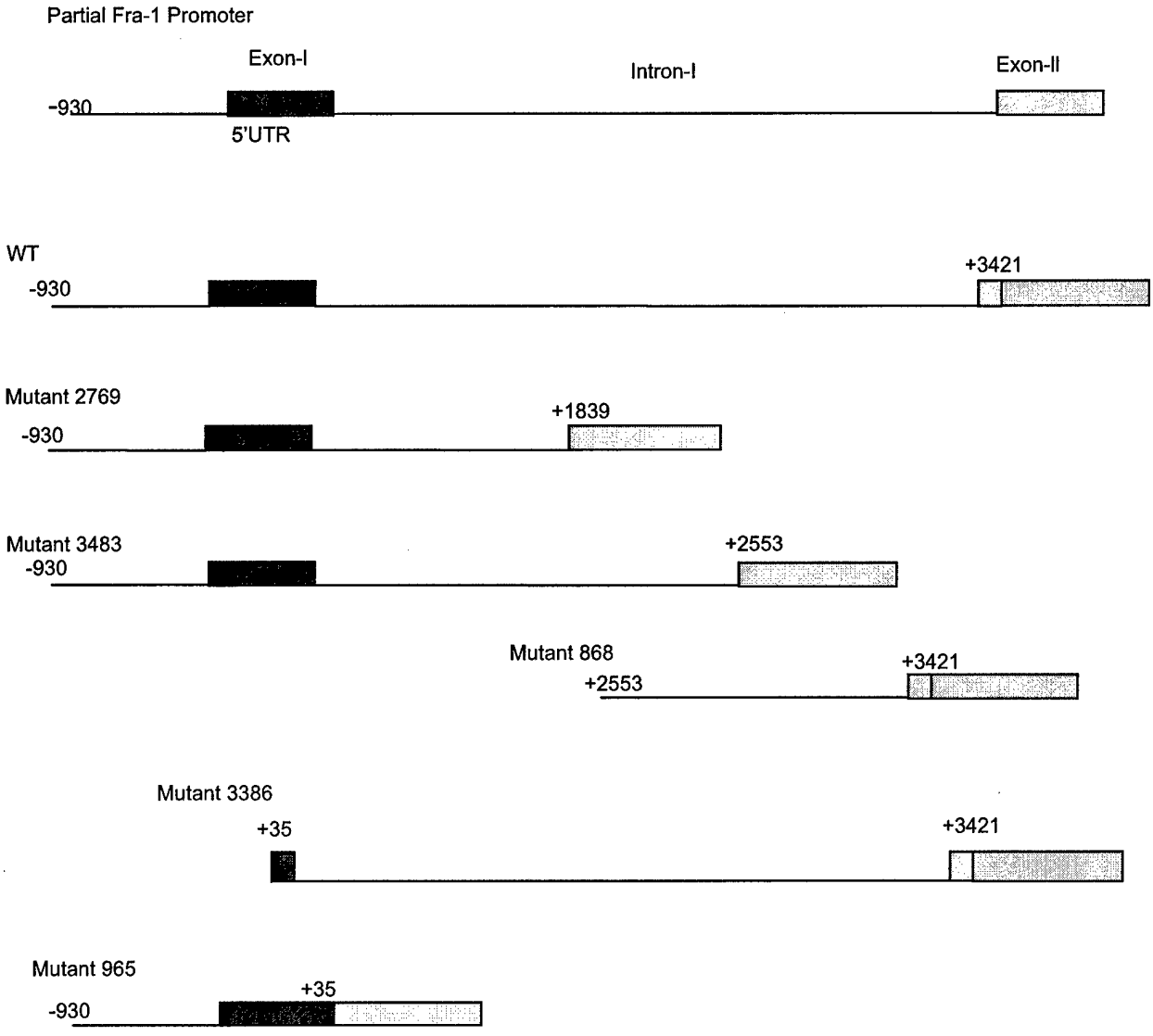



Figure 5

## A wild type Fra-1 promoter reporter construct faithfully Recapitulates the expression of the endogenous Fra-1 gene

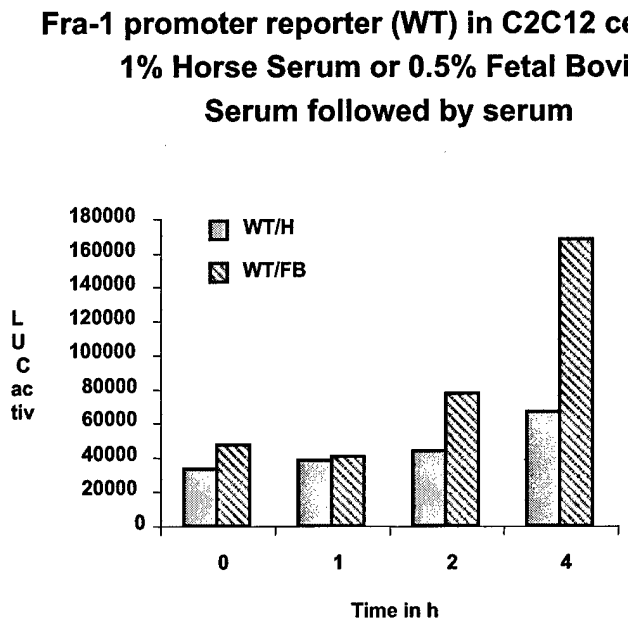
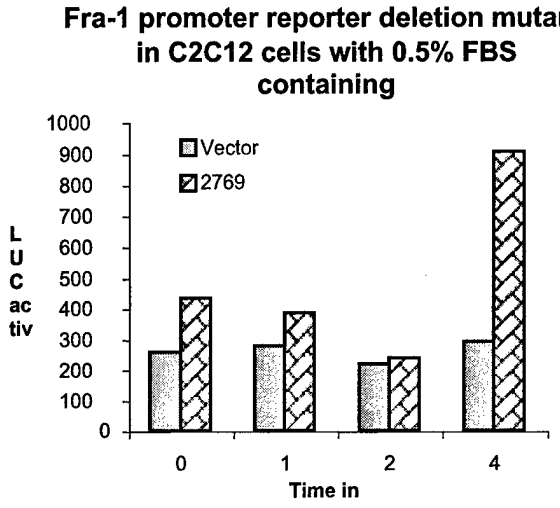
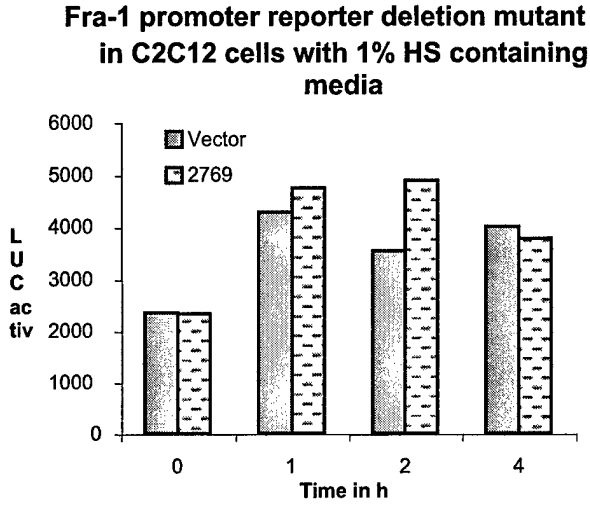
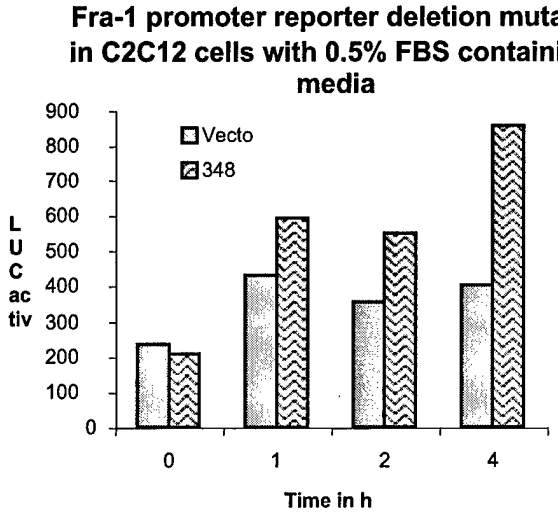



Figure 6

# Deletion mutants of Fra-1 promoter reporter construct showing activation of Fra-1 gene



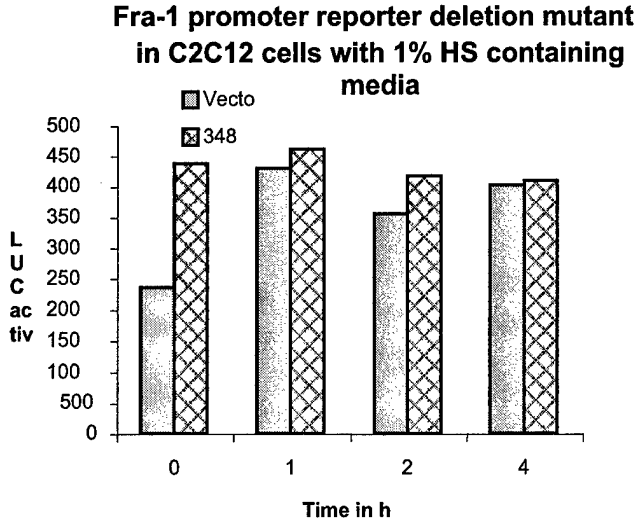




Figure 7

**Full-length Fra-1 promoter reporter showing a higher activation compare to deletion mutants construct upon serum stimulation in Rb+/+ myoblasts**

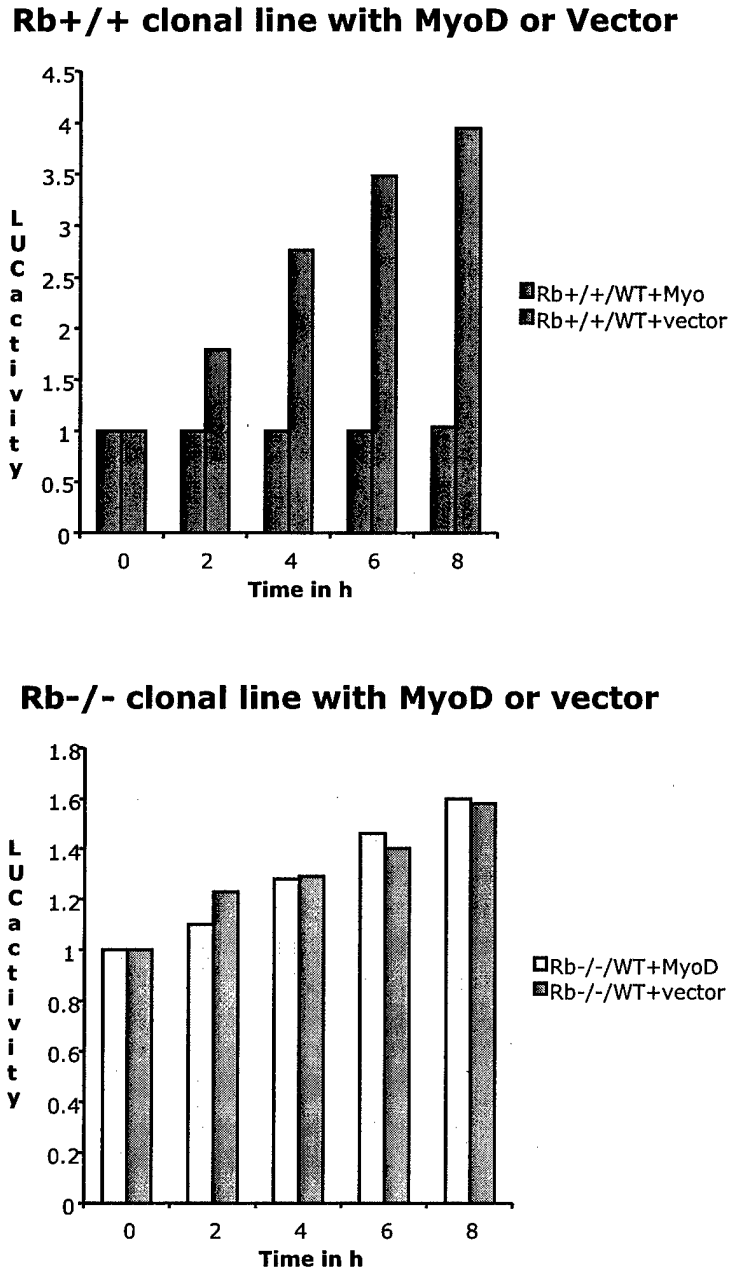



Figure 8

# Site directed mutagenesis for the conserved E-box element localized by Bayesian block alignment of intron-1 region between mouse and human Fra-1 promoter

Sequence homology from Intron-1 region of Fra-1 promoter

agaattcttagcagcctgtccgaggctgtccgtgtgttgctctggttgtccgtgtccct
tatccggtcaagtcctcatctctttgtgcgcagtatagagcccatgggccccaggcagt
gttccgaggggttcctggagaccacgaagtgttgggatgtgcgcggggt acctgcccg
ccacactcgcgctccacattctcggcacccgacgtctctcactgctggataggggcact
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gagatgcccctggcagtgcttctagcccag acgggggtcact agatgctgggtccccc
agtattgggctggggacatagctgtcca acttgcctaagcatgtgaggt cttc tgg
ctggaggggccccacatccttagctca agagcttgaacc agttttc ctcccaga cg

the E boxes and AP-1 sites are all in conserved

E box CANNTG

AP-1 site
CGGGTC

Figure 9

**PCR amplification of Fra-1 promoter fragment from DNA template generated by Chromatin Immunoprecipitation (ChIP) using MyoD specific antibody in C2C12 myoblasts**

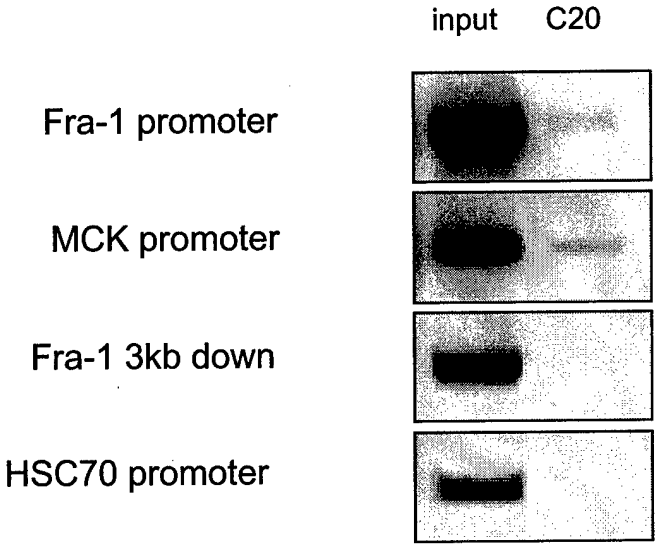



Figure 10

**PCR amplification of Fra-1 promoter fragment in Rb+/+ myoblasts with DNA template generated from Chromatin Immunoprecipitation (ChIP) using MyoD specific antibody**

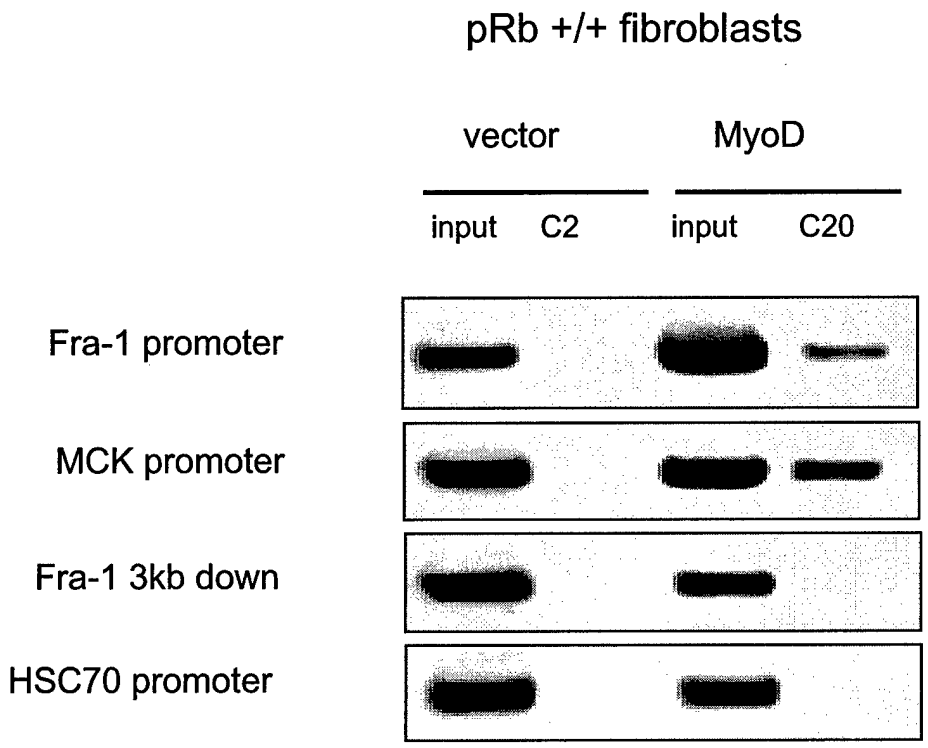
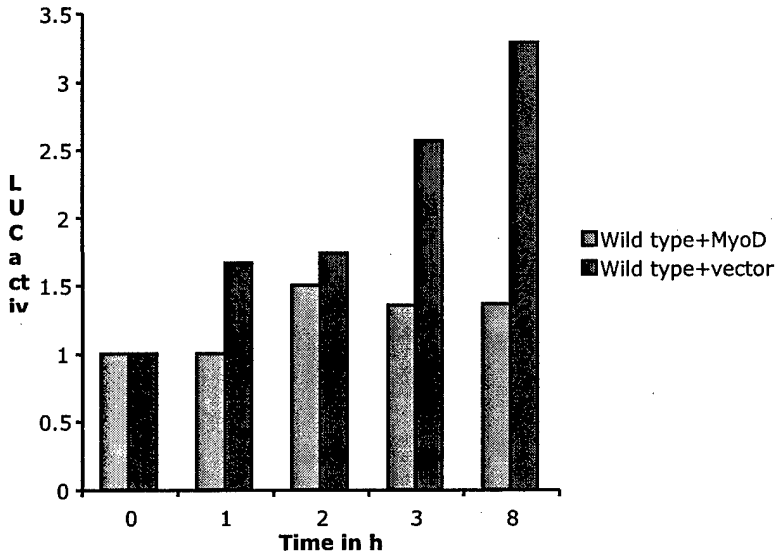



Figure 11

- Fra-1 promoter reporter analysis of wild type and E-box mutated stable lines of Rb+/+ myoblasts before and after serum stimulation

**Wild type clonal line with MyoD or**




**E-box-2 mutated clonal line with MyoD**

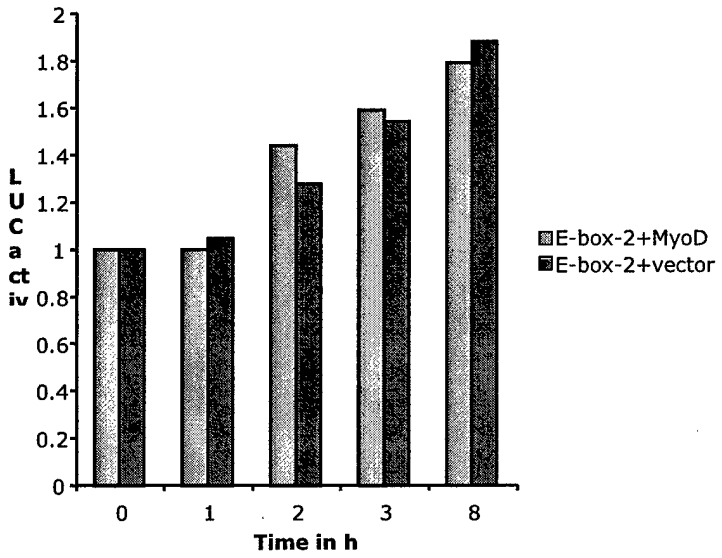



Figure 12